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Perinatal inhibition of NF-kappaB has long-term antihypertensive and renoprotective effects in Fawn-Hooded Hypertensive Rats

Maarten P. Koeners[1,‡], Sebastiaan Wesseling[1,2,‡], Manuel Sánchez[1,3,‡], Branko Braam[4] and Jaap A. Joles[1]

[1] Department of Nephrology and Hypertension, University Medical Center, Utrecht, Netherlands
[2] Division of Toxicology, Wageningen University, Wageningen, Netherlands
[3] Department of Pharmacology, School of Pharmacy, University of Granada, Spain
[4] Division of Nephrology and Immunology, Dept. Medicine, and Dept. Physiology
University of Alberta, Edmonton, Canada

‡ These authors contributed equally.

Running head: Perinatal PDTC in fawn-hooded hypertensive rats

Disclosure

None of the authors have any conflict of interest.

Key Words:

Developmental plasticity; hypertension; nuclear factor kappa B; renal hemodynamics; glomerulosclerosis

Address correspondence to:

Jaap A. Joles, D.V.M., Ph.D.
Department of Nephrology and Hypertension F03.223
University Medical Center Utrecht
P.O. Box 85500, 3508 GA Utrecht, Netherlands
Tel: +31 30 253 5269
Fax: +31 30 254 3492
Email: j.a.joles@umcutrecht.nl

Abstract

BACKGROUND Inhibition of transcription factor nuclear factor-kappa B (NFκB) is beneficial in various models of hypertension and renal disease. We hypothesized first that NFκB inhibition during renal development ameliorates hereditary hypertensive renal disease and next whether this was mediated via suppression of peroxisome proliferator-activated receptor (PPAR) γ coactivator 1α.

METHODS & RESULTS Prior to development of renal injury in Fawn-Hooded Hypertensive (FHH) rats, a model of hypertension, glomerular hyperfiltration, and progressive renal injury, NFkB activity, measured by nuclear protein expression of NFkB subunit p65, was enhanced twofold in 2d-old male and female FHH kidneys as compared to normotensive WKY rats ($p<0.05$). Treating FHH dams with PDTC, an NFκB inhibitor, from 2wk before birth to 4wk after birth diminished NFkB activity in 2d-FHH offspring to 2d-WKY levels ($p<0.01$). Perinatal PDTC reduced systolic blood pressure from 20wk onwards by on average 25 mmHg ($p<0.001$) and ameliorated proteinuria ($P<0.05$) and glomerulosclerosis ($p<0.05$). In kidneys of 2d, 2wk and adult offspring of PDTC-treated FHH dams, PPARγ coactivator 1α was induced on average by 67% (qPCR) suggesting that suppression of this factor by NFkB could be involved in renal damage. Follow-up experiments with perinatal pioglitazone, a PPARγ agonist, failed to confer persistent antihypertensive or renoprotective effects.

CONCLUSIONS perinatal inhibition of enhanced active renal NFκB in 2d FHH had persistent antihypertensive and renoprotective effects. However, this was not the case for PPARγ stimulation. NFkB stimulation is therefore involved in renal damage in the FHH model of proteinuric renal disease by pathways other than via PPARγ.

Introduction

Hypertension is associated with nitric oxide (NO) deficiency and increased reactive oxygen species (ROS).[1] ROS can activate nuclear factor-kappa B (NFκB) via redox-sensitive signal transduction. NFκB is as a general transcription factor for pro-inflammatory pathways.[2] Systemic and renal inflammation has been proposed to be an underlying mechanism which drives both hypertension development and renal injury.[3]

Both inflammation and upregulation of NFκB activation are already present in kidneys of 3-wk-old pre-hypertensive spontaneously hypertensive rats (SHR), and NFκB inhibition can prevent the development of hypertension.[4] Previously, we found in SHR that perinatal interventions, which manipulate the NO/ROS balance, can alleviate the development of hypertension in later life.[5-7] Therefore, it is conceivable that inhibiting NFκB sensitive pro-inflammatory pathways suppresses or prevents development of the hypertensive phenotype. Indeed, we found that perinatal treatment with an NFκB inhibitor (Pyrrolidine Di Thio Carbamate, PDTC) to SHR dams during pregnancy and lactation, abrogated development of hypertension in both female and male offspring, and lowered urinary excretion of lipid peroxides long after treatment was stopped.[8]

In the current study we used the Fawn-Hooded Hypertensive rat (FHH), in order to extend these observations beyond the SHR model. The FHH is a genetic model of hypertension with progressive renal injury characterized by mild systemic hypertension, marked proteinuria and glomerulosclerosis (GS).[9-11] Early initiation of anti-hypertensive treatment protects the FHH kidney more effectively than late initiation despite a similar anti-hypertensive effect.[12] Perinatal molsidomine, a tolerance-free NO donor, persistently decreased blood pressure and ameliorated renal injury in male and female FHH offspring.[13]

We hypothesized that NFκB activation is an early event that precedes development of hypertension and renal injury in FHH, and that perinatal inhibition of NFκB with PDTC ameliorates hypertension and is renoprotective in male and female FHH offspring. First, we studied NFκB activation in newborn FHH as well as direct and long-term effects on development of hypertension and renal damage of perinatal treatment with PDTC in female and male FHH offspring. Next, microarray studies were employed to screen the genome in the kidneys of newborn, young and adult FHH for genes persistently regulated in response to perinatal NFkB inhibition. Finally, since this analysis revealed that peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) gene expression was consistently increased in both genders and at all ages during and after perinatal PDTC treatment, we investigated the effects of perinatal treatment of FHH with the peroxisome proliferator-activated receptor-γ (PPARγ) agonist, pioglitazone on blood pressure and renal damage (proteinuria, histology) on male and female offspring.

Methods

Animals

Fawn-Hooded Hypertensive rats (FHH) were housed at 22^{o}C, humidity 60% and exposed to a 12 h light/dark cycle. FHH rats were obtained from our own colony, which was derived from the original colony at Erasmus University Rotterdam (FHH/EUR) maintained by Dr. A. Provoost. Normotensive Wistar-Kyoto (WKY) rats (Harlan-Olac Oxon, UK) were used as breeders exclusively to provide additional control pups for NFκB activity as measured by p65 levels. Sentinel animals were housed under the same conditions and regularly monitored for infection by nematodes and pathogenic bacteria and antibodies for rodent viral pathogens (International Council for Laboratory Animal Science, Nijmegen, Netherlands). The Utrecht University Board for studies in experimental animals approved the both PDTC and Pioglitazone protocols.

Treatment protocol, chronic follow up and terminal protocol

PDTC: From day 7 of gestation, dams and their offspring received either tap water (FHH con) or PDTC in drinking water (150 mg/L) until 4 wk of age (FHH PDTC). At 2d of age litters were culled to a maximum of 8 pups/litter and at 2wk of age to a maximum of 6 pups/litter in order to standardize drug intake during lactation. Kidneys of culled pups were used for renal NFκB activity and NFκB-sensitive gene expression studies (see below). Age and sex-matched WKY pups were also sacrificed at the same age for measurement of renal NFκB activity. In each subgroup (FHH or WKY, male or female, 2d or 2wk) 5 pups were used from 5 different litters. The remaining FHH pups were weaned at 4wk and used for chronic follow-up until 36wk in males and 42wk in females (see protocol below).

Pioglitazone: During the study with pioglitazone we followed the same treatment protocol. Starting at day 7 of gestation and until 4wk of age, FHH dams and their offspring received powdered chow (FHH Con) or pioglitazone (10 mg/kg body weight of the dam; FHH Pio) mixed with the powdered chow. At 2d of age litters were culled to a maximum of 8 pups/litter and at 2wk of age to a maximum of 6 pups/litter in order to standardize pioglitazone intake during lactation. Livers of culled pups at those two ages were used for PPARγ-sensitive gene expression (see below). The remaining FHH pups were weaned at 4wk and used for chronic follow-up until 42wk (see protocol below).

At 10wk of age 10 FHH Con and 10 FHH Pio rats were used for glomerular counting, as described below. Standard protocols and procedures for sample collection were followed during the terminal experiments with the remaining FHH Con (n=14) and FHH Pio (n=15) male and female rats at 42wk of age as described below.

Renal NFκB activity in pups: Whole snap-frozen kidneys from male and female FHH and WKY pups (2d and 2wk) were homogenized and the extracts were isolated using the Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the protocol of the supplier. Concentration of protein extracts was determined by the Bradford method (BioRad Laboratories, Veenendaal, Netherlands). The NFκB component p65 was measured using TransAM™ NFκB p65 (Active Motif). A dilution range of recombinant NFκB p65 protein (Active Motif) was used to quantify p65 in the samples. To confirm linearity of the dilution range (correlation r^2=0.99), the positive control cell extract included in the TransAm™ kit and several random samples were also diluted. All samples were measured in duplicate. The relative p65 content in nuclear extract was determined by comparing the p65 to protein concentration ratio of the same sample.

Chronic follow-up: Systolic blood pressure (SBP) was measured regularly from 4wk of age by tail-cuff. After SBP measurement rats were placed in metabolism cages without food for 24h, but with free access to tap water with 2% glucose, for determination of urinary excretion of protein, measured with Coomassie blue (Bradford).[14] In the PDTC experiment female offspring were studied till 42wk and male offspring till 36wk. Females were followed 6wk longer because male FHH generally develop proteinuria sooner than female FHH.[13] In the Pio experiment all offspring were studied till 42wk.

Terminal protocol: On the day of the experiment surgery was performed as described.[15-17] In short, rats from the PDTC experiment were anesthetized with intraperitoneal pentobarbital sodium 60 mg/kg body weight. Rats from the Pioglitazone experiment were anesthetized through inhalation of isoflurane 2%.[16] This discrepancy was dictated by altered ethical board requirements. Renal function was assessed through p-amino hippuric acid (PAH) and inulin clearances. We used PAH clearance to determine the renal plasma flow (RPF) and inulin clearance to determine glomerular filtration rate (GFR).[13,16] RBF was calculated as RPF/(1-hematocrit).

Microarray screening for genes in the kidney responsive to NFkB inhibition

Detailed methodology for the microarray approach can be found in the “Supplementary Data”. Kidneys from 2 days, 2 weeks and adult FHH of both genders were used (n=5/group). Data processing was performed as described.[18] The microarray data set was submitted as MIAME through NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE57066.

Real-time quantitative PCR

To determine the direct effects of maternal treatment with a NFκB inhibitor or a PPARγ agonist on mRNA expression of NFκB- or PPARγ-target genes, RNA was isolated from kidney and liver of 2d

and 2wk old offspring, reverse transcribed to cDNA and quantified using real-time quantitative PCR (qPCR). Real-Time qPCR was performed on a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA). The following TaqMan® Gene Expression Assays (Applied Biosystems) were used: NFκB-target genes: PGC-1α (Rn01453111_m1), PPARγ-target genes: Adiponectin (Rn00595250_m1), Sirtuin 6 (Rn01408250_g1), Foxo-1 (Rn01494868_m1) and CISD-1 (Rn01429087_m1); and housekeeping genes: ß-actin (Rn00667869_m1) and calnexin (Rn00596877_m1). Reactions were carried out in duplicate. Cycle time (Ct) values for genes of interest were normalized for mean Ct-values of Calnexin and β-actin, which we previously determined to be the two most stable housekeeping genes across all groups using Normfinder (http://moma.dk/normfinder-software) and GeNorm (http://medgen.ugent.be/~jvdesomp/genorm/), and expressed using the ΔΔCt-method. Hence, steady state mRNA levels in treated FHH were expressed as % change relative to age- and gender-matched control FHH.

Morphology

At the end of each terminal experiment the right kidney was harvested, blotted dry, weighed, snap frozen and stored at -80^{o}C. Subsequently, left kidney preservation was done as described by Black et al.[19] Heparin sodium (1 unit heparin/gram body weight), to prevent clotting, and papaverine hydrochloride (1.2 mg/rat), to dilate the vasculature were administered via femoral artery. After approximately 3 min the abdominal aorta was exposed and tied off above the left renal artery. Then the kidney was perfused in situ with saline before fixation with 2.5% glutaraldehyde (w/v) in 0.1M phosphate buffer (pH 7.4) at a pressure of 10 mmHg above measured MAP for approximately 3 min, excised, de-capsulated, and placed in 4% buffered formaldehyde.

Paraffin sections were stained with periodic acid-Schiff. Glomeruli were counted at magnification 50x, by applying a grid on randomly chosen fields, and expressed as number of glomeruli/mm^3 (n), calculated by the formula n=G/(F x A x (D+T)), were G is the number glomeruli counted, F the number of fields counted, A the grid area, D, the average glomerular tuft diameter, and T the section thickness (0.004mm).[20] All glomeruli were counted, irrespective of any sign of injury. Fifty glomeruli were scored for the presence of sclerotic lesions.[21] We differentiated full sclerotic and partial sclerotic glomeruli and the sum of these two (sum). Glomerular and tubulo-interstitial ED-1-antigen-positive monocytes/macrophages were determined as described.[14] Paraffin sections (3 μm) of formaldehyde-fixed kidney were deparaffinized and rehydrated. Incubation with the ED-1 mouse monoclonal antibody (Serotec/Camon, Wiesbaden, Germany) demonstrated monocytes/macrophages. After application of ED-1 (dilution 1: 2500 in PBS containing 5% BSA, 0.4% sodium azide) to the slides at 22 ^{o}C for one hour, bound antibody was detected by the DAKO EnVision ^{R} + System (prediluted peroxidase-dextran-conjugated goat anti-mouse antibody and DAB colour reaction).

Statistics

Values are expressed as mean ± SEM. Data were compared with unpaired t-test, one-way ANOVA and two-way ANOVA for repeated measurements where appropriate. The Student-Newman-Keuls test was used as a post-hoc test ($p<0.05$). Significance of differentially expressed genes between FHH PDTC vs. FHH Con was determined by Cyber T test using Log_2 transformed and quantile normalized data ($P<0.05$).

Results

Perinatal NFκB inhibition

NFκB activity in pups: Renal levels of NFκB component p65 (mg/g protein) were increased in both female and male 2d-old FHH pups when compared to age and gender matched WKY pups (on average +50% and +200% in females and males, respectively; both P<0.01, Figure 1). Maternal treatment with PDTC in FHH completely prevented this increase in both females and males (both P<0.01). At 2w of age there were no longer differences in renal p65 levels between FHH and WKY and there was also no effect of PDTC in FHH.

Development: All litters were carried to full gestation and litter size was not affected by PDTC (FHH Con: 7 ± 1 pups/litter, FHH PDTC: 7 ± 1 pups/litter). Perinatal PDTC (until 4w) resulted in a mild but significant decrease in body weight from 12w in females and males until sacrifice (Supplementary Fig. 1 and Table 1).

Blood pressure, proteinuria, glomerular injury and glomerular density: Perinatal PDTC treatment reduced SBP at 4w in females and males and had a pronounced long-term anti-hypertensive effect resulting in a persistently reduced SBP by 20-30 mmHg in both female and male FHH until sacrifice (p<0.001, Fig. 2). Perinatal PDTC reduced proteinuria from 20w onwards in male FHH and at 28w and 36w in female FHH (Fig. 3) and also reduced GS at the end of the follow-up period. Full sclerotic glomeruli and the sum of partial and full sclerotic glomeruli were significantly decreased by Perinatal PDTC in both female (p<0.05) and male (p<0.001) FHH (Fig. 4). Glomerular macrophages were reduced by perinatal PDTC in males (1.4±0.3 vs. 7.0±1.1 ED1-positive cells/100 glomeruli, p<0.05) as were tubulo-interstitial macrophages (0.7±0.1 vs. 2.4±0.5 ED1-positive cells/field, p<0.05). Glomerular macrophages were practically absent in all females, but tubulo-interstitial macrophages were reduced by perinatal PDTC in females (0.04±0.03 vs. 0.65±0.30 ED1-positive cells/field, p<0.05). Glomerular density was increased in male FHH PDTC (p<0.001, Supplementary Fig. 2) but not in female PDTC.

Renal hemodynamics at the end of follow up: MAP assessed by direct femoral artery pressure measurements confirmed significantly reduced arterial pressure in both female and male FHH at the end of the follow-up period (p<0.05 and P<0.01, respectively; Table 1). Perinatal PDTC decreased GFR in female FHH (p<0.01) and decreased GFR, RPF and RBF in male FHH (all p<0.05, Table 1).

Renal PDTC sensitive gene expression in pups: Differential gene expression was studied with Illumina Bead Arrays in whole kidney homogenates of 2d-, 2w and adult male and female FHH offspring of dams treated with PDTC in comparison to age and sex-matched offspring of untreated

FHH dams (n=5/group). At 2d of age the signal on the microarray of 349 genes was different in the offspring of PDTC treated animals compared to matched offspring of untreated animals. This suggested induction of the expression of 276 genes and suppression of 73 genes in both male and female offspring. At 2w of age, only 9 PDTC sensitive genes were similarly differentially expressed, i.e. up (n = 7) and down (n = 2). None of these 9 genes were differentially expressed at 2d of age. The low number of PDTC-sensitive differential gene expression as studied by microarray coincides with the absence of differences in renal NFκB component p65 at 2w (Fig. 1). In adults, 25 PDTC sensitive genes were similarly differentially expressed, i.e. up (n = 18) and down (n = 7). None of these 25 genes were differentially expressed at 2w of age.

The most consistently regulated NFκB-sensitive gene was peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α) (Table 2). This gene showed increased expression during and after PDTC treatment, although the increase was not significant at 2wk in males. This was confirmed by qPCR, although in this case the difference was not significant at 2d (Table 2). A selection of other NFκB-sensitive genes, grouped according to function, is listed in Supplementary Table 1. Functional groups include Regulation of NFκB (Nkiras1), insulin-like growth factors (IGF2 and IGFbp3), angiogenesis and nitric oxide (VEGF, Endothelin B receptor) and nephrogenesis (Nidogen1, Notch1, and Nephrin). Many of the other differentially expressed genes were related to cell-cell communication and extracellular matrix. Note that practically all these genes were consistently differentially upregulated by NFκB inhibition at 2d of age. Notably, deoxyribonuclease 1 (Dnase1) was markedly down-regulated.

Perinatal PPARγ stimulation

Hepatic PPARγ activity in pups: The liver is the canonical target of PPARγ.[22,23] Hepatic mRNA expression of PPARγ downstream genes showed increased expression of CSD-1 and SIRT-6 genes at 2d-old FHH rats perinatally treated with Pio compared with age-matched FHH control rats. At 2wk the pattern shifted and we found decreased expression of AdipoQ and FOXO-1 genes after perinatal treatment with Pio (Table 3).

Development: All litters were carried to full gestation and litter size was not affected by perinatal Pio (FHH Con: 9 ± 1 pups/litter, FHH Pio 10 ± 1 pups/litter). Perinatal Pio resulted in no change in body weight in female offspring but a significant and persistent decrease in body weight from 24wk in male offspring (Supplementary Fig. 3).

Blood pressure, proteinuria, glomerular injury and glomerular density: Perinatal Pio treatment reduced SBP at 4w in females and males. However, there was no persistent long-term anti-hypertensive effect (Supplementary Fig. 4). Perinatal Pio had no significant effects on proteinuria in

either female or male FHH (Supplementary Fig. 5), nor was there a reduction in GS in female or male Pio offspring at 42w (Supplementary Fig. 6). Glomerular density at 10w and 42w was unaffected by perinatal Pio (Supplementary Fig. 7).

Renal hemodynamics at the end of follow up: MAP was also not significantly affected at the end of the follow-up period (Table 4). Perinatal Pio had no effect on GFR, RPF and RBF in female FHH but decreased GFR, RPF and RBF in male FHH (all $p<0.05$, Table 4). RVR was unchanged in FHH Pio females but increased in FHH Pio males ($P<0.05$). Note that the FHH Con data in Table 4 differ slightly from those in Table 1, primarily due to differences in the anaesthetic regimen.

Discussion

The present study addressed the question whether the previously observed induction of the pro-inflammatory transcription factor NFkB in the early life of the SHR was also present in the FHH, whether inhibiting NFkB in the perinatal situation attenuated the hypertension and renal damage in the FHH and whether this was related to depressed PPAR**γ**. In the FHH, NFkB is induced early in life and perinatal inhibition of NFkB with PDTC indeed ameliorated the hypertension and renal injury. Gene expression analysis of the kidney cortex revealed PPAR**γ** depression as a potential mechanism of NFkB mediated hypertension and renal injury in the offspring. Despite sex specific alterations in renal hemodynamics, perinatal administration of a PPAR**γ** agonist did not ameliorate hypertension and renal damage.

Activation of redox sensitive transcription factor NFκB is associated with hypertension, with renal injury or both[2] Moreover, inhibition of NFκB with PDTC can prevent or ameliorate both hypertension and renal injury in many models.[4,24-28] Previously, we documented that perinatal interventions affecting the redox balance can ameliorate hypertension in SHR.[5,6,29,30] We also documented that in FHH, a model of hypertensive renal injury [9-11] perinatal administration of an NO donor ameliorates hypertension and renal injury.[13] In the current study, we first addressed the question whether NFκB is already activated during nephrogenesis in the FHH model and whether such activation plays a role in subsequent renal disease and hypertension. We could indeed demonstrate activation of NFκB in kidneys of newborn FHH, by increased levels of p65, a component of active NFκB.[31] Activation of NFκB was not measured in adult rat because we probed effects relating to development not injury. Moreover, glomerular density was substantially increased in adult male FHH PDTC as compared to FHH Con. Perinatal NFκB inhibition persistently ameliorated the development of hypertension and proteinuria in both female and male FHH. At the end of the study GFR and GS were both reduced, suggesting persistent amelioration of hyperfiltration by Perinatal PDTC. In the FHH rat, prone as it is to hyperfiltration, [9,11,13] a similar hemodynamic response was observed after perinatal administration of a NO donor.[13] Therefore, in this model of hereditary hypertension associated renal injury, early life NFkB activation seems pivotal to develop hypertension and renal injury. These initial results support a role for persistent alterations in hemodynamics after perinatal PDTC in FHH. Note that in SHR, a strain with increased RVR,[15,17] perinatal PDTC did not significantly affect GFR or RVR in either female or male offspring.[8]

Proteinuria was ameliorated by perinatal PDTC in conjunction with a significant blood pressure reduction. Moreover, development of glomerular sclerosis was clearly reduced by perinatal PDTC treatment, suggesting that the less pronounced incline in proteinuria observed in FHH PDTC, represents less progressive renal injury. In FHH controls RBF and GFR were positively correlated

with sclerosis.[13] Probably this illustrates low preglomerular resistance in the FHH strain, resulting in glomerular hypertension, and consequently glomerular hyperperfusion, hyperfiltration and injury. Our current findings are compatible with the idea that perinatally inhibiting the transcription factor NFκB can prevent the development of glomerular hypertension by increasing afferent arteriolar resistance (since both GFR and RBF decrease). The reduction in macrophage infiltration was probably secondary to the reduction in glomerular proteinuria.[13] That said, the other possibility is that non-hemodynamic mechanisms related to activation of NFkB early in life related genes are responsible for the hypertension and renal damage in the FHH. Generally PDTC is applied as an NF-kappa B inhibitor. However, antioxidant effects have also been described[32-34], although arguably these could be secondary to NF-kappa B inhibition. More specific "off-target" effects include Nrf2 activation leading to induction of glutamate cysteine ligase modulatory gene expression[35] and stromelysin expression via a tyrosine kinase-AP-1 pathway.[36] Therefore, we feel that perinatal targeting of NF-kappa B by PDTC in our studies in SHR[8] and FHH (this study) should be seen as proof of principle experiments in models of genetically programmed hypertension (SHR & FHH) and renal injury (FHH), and certainly not as a direct incentive to apply PDTC in the obstetric or neonatal clinic.

Because maternal treatment with the NFκB inhibitor PDTC normalised renal levels of the NFκB component p65, we assessed NFκB-sensitive renal gene expression in the offspring. At 2d of age, in the nephrogenic phase, NFκB-sensitive renal gene expression was characterized by a pronephrogenic and angiogenic pattern. Nephrogenesis in the rat starts at embryonic day 12 and is complete at about 10 days after birth.[37] NFκB activity, i.e. p65 levels, and NFκB-sensitive gene expression are increased in the middle of this process in the FHH. Parallel with p65 levels, NFκB-sensitive gene expression waned at 2w of age, suggesting an association between NFκB activation and nephrogenesis in FHH. Indeed, Nidogen1, Notch1, Nephrin and VEGF, genes that are known to be involved in nephrogenesis [38,39] and angiogenesis [40] were differentially expressed at 2d in female and male offspring. Specifically, peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) was differentially expressed. This transcription factor has powerful anti-inflammatory and vasculogenic effects and is known for its stimulatory effects on all three PPAR's: α, ß, and γ. [41,42] Several other groups of genes of interest were identified by this discovery approach. These groups included insulin signaling, and induction of a large number of genes related to cell-cell communication and extracellular matrix (Supplementary table 1). All in all this gene expression analysis emphasized the importance of angiogenesis and nephrogenic genes and cell-cell communication and extracellular matrix. In line with the induction of nephrogenic genes is the observation of ~25% increase in glomerular density in adult male FHH treated perinatally with PDTC. Since we previously could identify candidates for perinatal treatment in SHR using differential gene expression of renal homogenates,[29,30] in follow-up experiments we now tested

whether depression of PPARγ, downstream of the transcription factor PGC-1α, could be a mediator of hypertension and renal injury in FHH rats.

Perinatal treatment with the PPAR-γ agonist pioglitazone (Pio) did not persistently reduce arterial pressure, proteinuria or glomerulosclerosis in FHH suggesting that other factors downstream of NFκB confer renal protection. Exposure of the pups to Pio was confirmed by characteristic changes in hepatic gene expression after PPARγ agonists.[22,23] In addition, there appeared to be a direct antihypertensive effect of Pio at 4wk in female and male FHH. Mild direct antihypertensive effects of PPARγ agonists have been reported in models of genetic or induced hypertension,[43-46] including perinatal low-protein diet.[47] Moreover, the late appearance (after 5-6 months of age) of reduced body weight in FHH Pio males, suggested metabolic programming. Lower body weight and improved glucose tolerance was observed after two weeks of Pio in a murine model of programmed obesity.[48] Interestingly, there was a reduction in hyperfiltration and hyperperfusion in the male, but not the female FHH at 42 weeks after Perinatal Pio. Specifically in the male FHH, renal hemodynamic changes reflected the changes in the PDTC treated offspring, although the 9 mmHg decrease in MAP was not significant. The simultaneous decrease in GFR and RPF is compatible with an increase in afferent arteriolar resistance. Interestingly therefore is that both perinatal PDTC and perinatal Pio affected renal hemodynamics (although obviously this was less pronounced in Pio treated rats), yet only perinatal PDTC could dampen development of hypertension and renal injury. This suggests that hemodynamic factors may not be the crucial link between perinatal induction of NFkB and subsequent renal damage. In future experiments, it might be interesting to further probe the PGC-1α pathway with a PPAR-ß agonist. PPAR-ß agonists have been shown to have antihypertensive and renoprotective effects in genetic and acquired models of hypertension.[49,50]

In conclusion, these data indicate that renal NFκB expression is enhanced in 2d FHH and that this can be ameliorated by the NFkB inhibitor PDTC. Perinatal PDTC stimulates gene expression related to angiogenesis and nephrogenesis, and has persistent antihypertensive and renoprotective effects, coinciding with less hyperfiltration. A key downstream layer seems the PGC-1α pathway, yet perinatal PPARγ stimulation did not affect hypertension and renal damage. Interestingly, renal hemodynamics effects of perinatal PPARγ and perinatal PDTC were similar in male offspring. We conclude that in the FHH rat early induction of NFkB is involved in blood pressure regulation and glomerular damage in the offspring, yet, when we tested one of the downstream genes of the PGC-1α pathway, PPARγ, the findings also pointed towards non-hemodynamic pathways for protection.

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Figure legends

Figure 1 Renal NFκB activity assessed by p65 levels in 2d- and 2w-old female and male FHH rats, FHH rats treated with PDTC (via their dams), and WKY rats.
$ $p<0.01$ vs. WKY and ** $p<0.01$ vs. FHH control

Figure 2 Systolic blood pressure in female and male FHH Con (open triangles) and FHH PDTC (closed triangles).
* $p<0.05$ vs. FHH Con

Figure 3 Urinary protein excretion in female and male FHH Con (open triangles) and FHH PDTC (closed triangles).
* $p<0.05$ vs. FHH Con

Figure 4 Full, partial and sum glomerular sclerosis (GS) incidences in female (panel A) and male (panel B) FHH. Con (white bars) and FHH PDTC (black bars).
* $p<0.05$ and ** $p<0.01$ vs. FHH Con

Table 1: Renal function (left plus right kidney) in adult female and male FHH Con and FHH PDTC

	FHH Con	**FHH PDTC**	**p-value**
Females 42wk **final N** (pups/litters)	10/7	8/5	
Body weight, g	277 ± 4	258 ± 5	0.008
Right kidney wt, g/100 g BW	0.43 ± 0.01	0.41 ± 0.01	NS
MAP, mmHg	114 ± 3	104 ± 2	0.016
GFR, ml/min	3.34 ± 0.20	2.41 ± 0.11	0.002
ERPF, ml/min	9.77 ± 0.90	7.58 ± 0.60	0.068
Hematocrit, vol/vol	0.42 ± 0.01	0.43 ± 0.01	NS
RBF, ml/min	16.93 ± 1.63	13.23 ± 1.03	0.083
RVR (MAP/RBF), units	6.63 ± 0.66	8.20 ± 0.68	NS
Males 36wk **final N** (pups/litters)	10/5	16/6	
Body weight, g	407 ± 5	386 ± 4	0.005
Right kidney wt, g/100 g BW	0.37 ± 0.01	0.33 ± 0.01	0.007
MAP, mmHg	122 ± 2	115 ± 2	0.006
GFR, ml/min	3.41 ± 0.14	2.96 ± 0.15	0.047
ERPF, ml/min	10.68 ± 0.68	8.85 ± 0.42	0.025
Hematocrit, vol/vol	0.47 ± 0.01	0.47 ± 0.01	NS
RBF, ml/min	20.04 ± 1.34	16.55 ± 0.67	0.016
RVR (MAP/RBF), units	6.41 ± 0.54	7.08 ± 0.01	NS

mean ± SEM, t-test

Table 2: Differential peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α) gene expression in female and male FHH PDTC *versus* FHH Con

	females			males		
	microarray	qPCR		Microarray	qPCR	
	FHH PDTC / FHH Con	Con	PDTC	FHH PDTC / FHH Con	Con	PDTC
2 days	149*	100 ± 69	142 ± 65*	145*	100 ± 85	127 ± 68
2 weeks	127*	100 ± 70	187 ± 49*	114	100 ± 111	170 ± 67*
adults	126*	100 ± 100	236 ± 54*	139*	100 ± 86	138 ± 53*

Differential expression was determined in the microarray by Cyber t-test and in the qPCR (% ± SEM) by t-test (* $P<0.05$ vs. age-matched FHH Con set at 100%). n=5/group

Table 3: Differential hepatic PPARγ target gene expression in FHH Pio *versus* FHH Con pups (pooled female and male data)

	CSD-1	**SIRT-6**	**AdipoQ**	**FOXO-1**
2 days	125 ± 11*	129 ± 8*	13 ± 50	122 ± 14
2 weeks	96 ± 21	84 ± 15	10 ± 24*	65 ± 18*

Group size: 2d FHH Con n = 10, 2d FHH Pio n = 10, 2w FHH Con n = 12, 2w FHH Pio n=9.

Differential expression was determined by qPCR (% ± SEM) by t-test (*p ≤ 0.05 vs. age-matched FHH Con set at 100%). mean ± SEM

Table 4: Renal function in female and male FHH Con and FHH Pio

	FHH Con	**FHH Pio**	**p-value**
Females 42wk **final N** (pups/litters)	10/5	8/5	
Body weight, g	285 ± 5	276 ± 5	NS
Right kidney wt, g/100 g BW	0.49 ± 0.02	0.48 ± 0.01	NS
MAP, mmHg	127 ± 5	125 ± 3	NS
GFR, ml/min	1.67 ± 0.24	1.51 ± 0.15	NS
ERPF, ml/min	6.85 ± 0.86	5.74 ± 0.45	NS
Hematocrit, vol/vol	0.36 ± 0.01	0.35 ± 0.01	NS
RBF, ml/min	10.6 ± 1.4	8.9 ± 0.7	NS
RVR (MAP/RBF), units	13.8 ± 2.2	14.5 ± 1.3	NS
Males 42wk **final N** (pups/litters)	7/5	11/5	
Body weight, g	454 ± 13	425 ± 8	0.006
Right kidney wt, g/100 g BW	0.41 ± 0.01	0.42 ± 0.01	NS
MAP, mmHg	108 ± 3	99 ± 4	NS
GFR, ml/min	2.32 ± 0.30	1.58 ± 0.13	0.024
ERPF, ml/min	10.25 ± 1.08	6.72 ± 0.80	0.019
Hematocrit, vol/vol	0.37 ± 0.01	0.39 ± 0.007	NS
RBF, ml/min	16.0 ± 1.5	11.0 ± 1.3	0.029
RVR (MAP/RBF), units	7.0 ± 0.5	9.5 ± 0.7	0.020

mean ± SEM, t-test

Figure 1

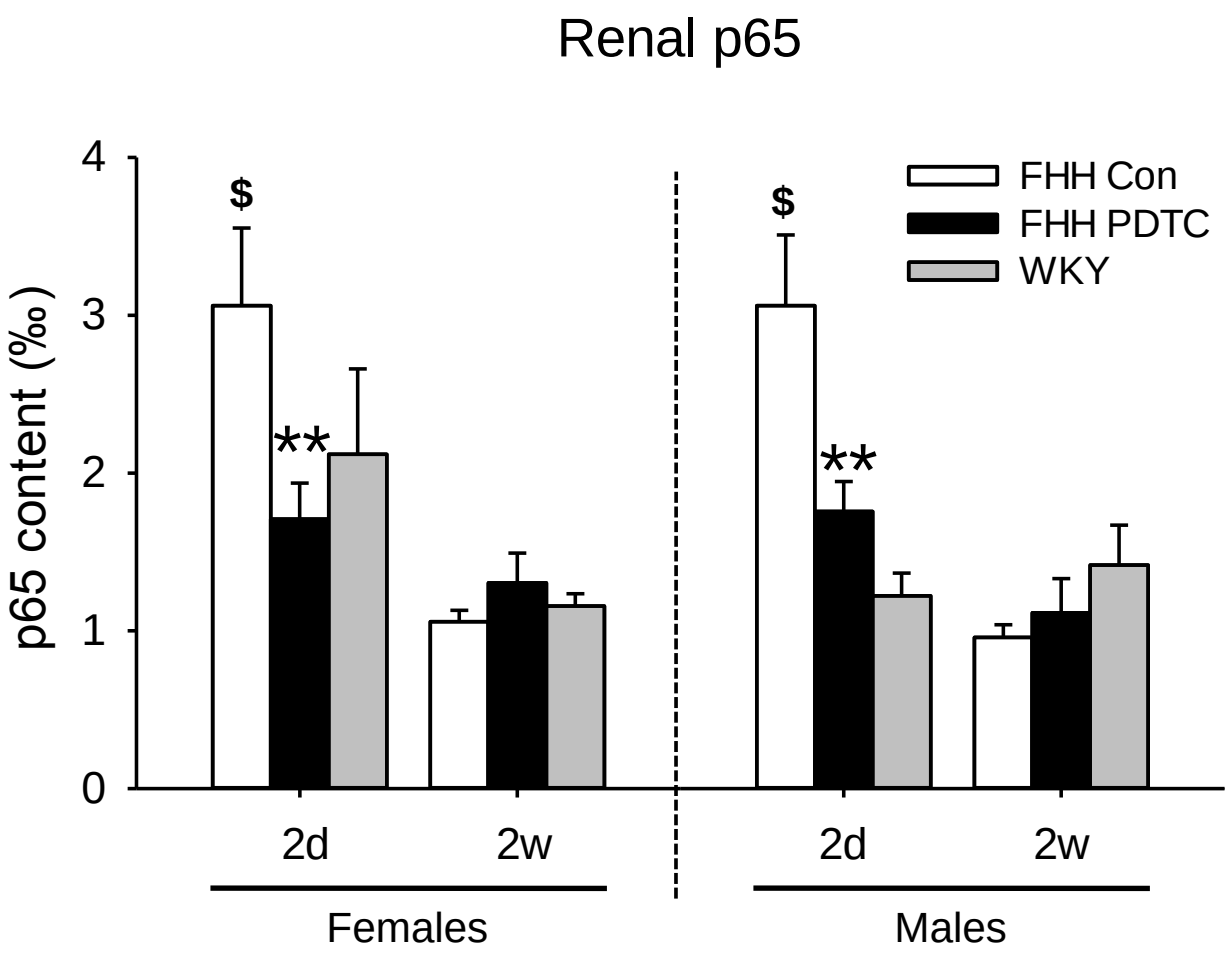

Renal p65
p65 content (‰)
4
3
2
1
0
$
**
$
**
FHH Con
FHH PDTC
WKY
2d
2w
2d
2w
Females
Males

Figure 2

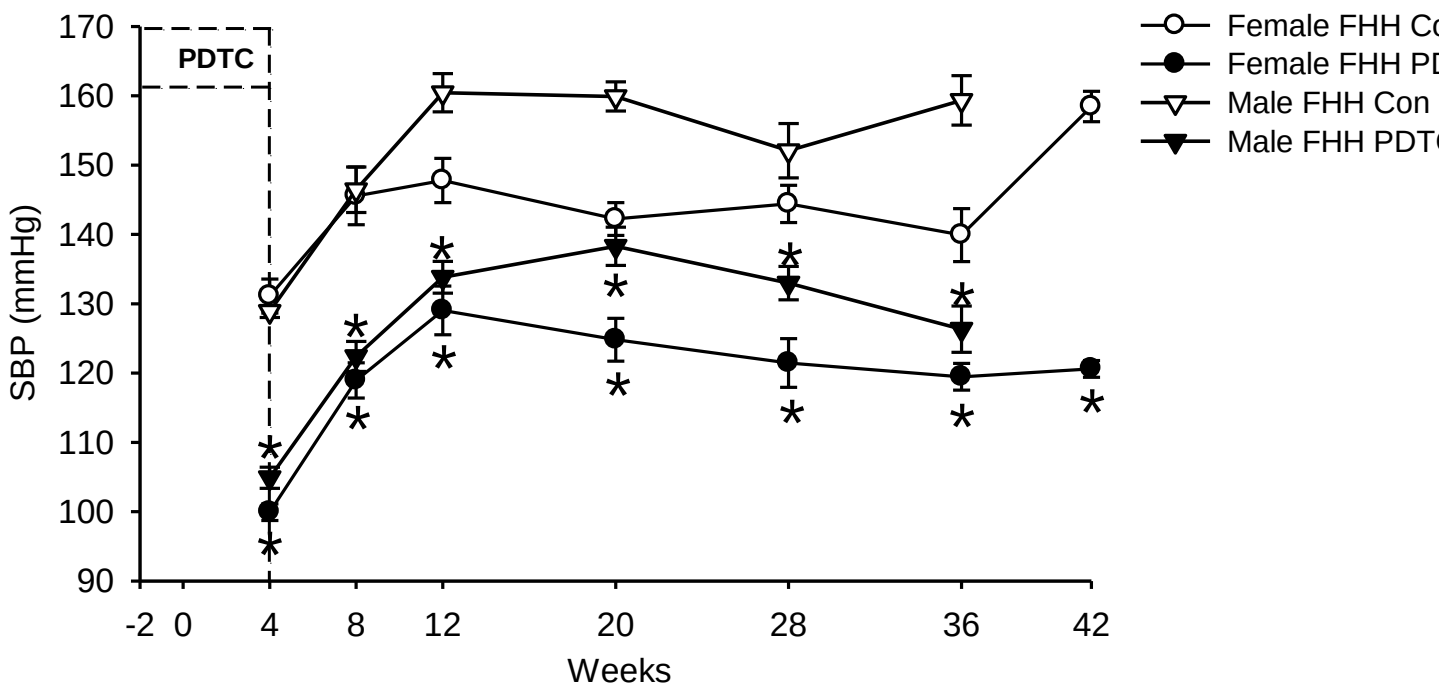

PDTC
SBP (mmHg)
170
160
150
140
130
120
110
100
90
-2
0
4
8
12
20
28
36
42
Weeks
Female FHH Co
Female FHH PD
Male FHH Con
Male FHH PDT

Figure 3

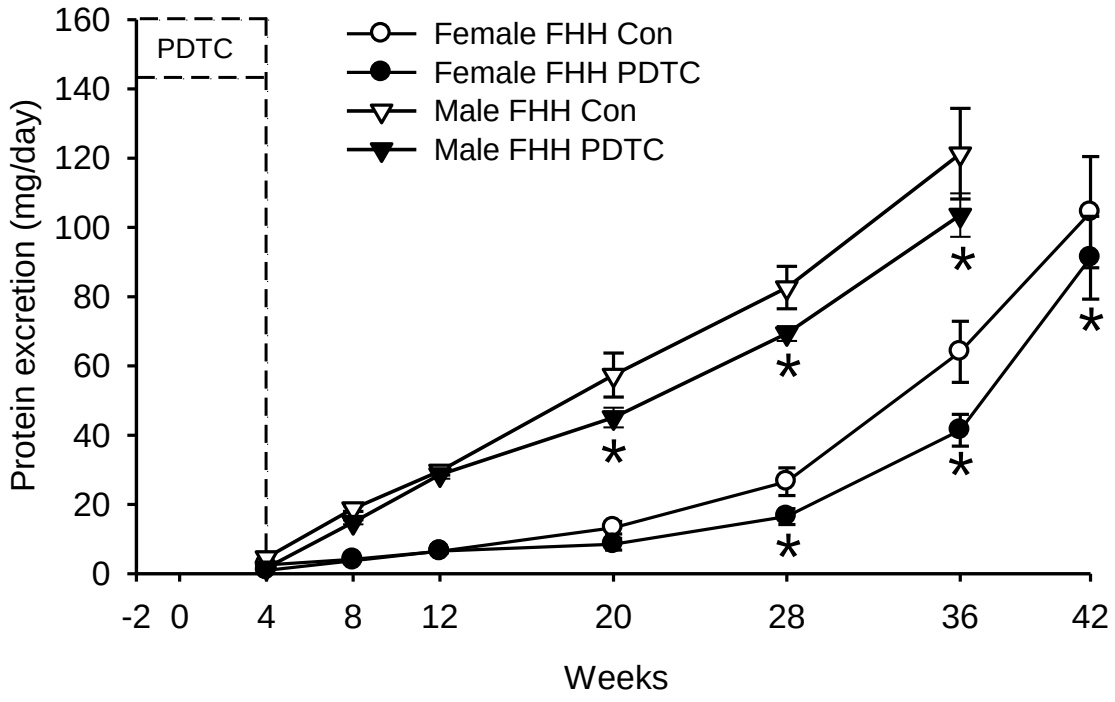

PDTC
Female FHH Con
Female FHH PDTC
Male FHH Con
Male FHH PDTC
Protein excretion (mg/day)
160
140
120
100
80
60
40
20
0
-2
0
4
8
12
20
28
36
42
Weeks
*
*
*
*
*

Figure 4

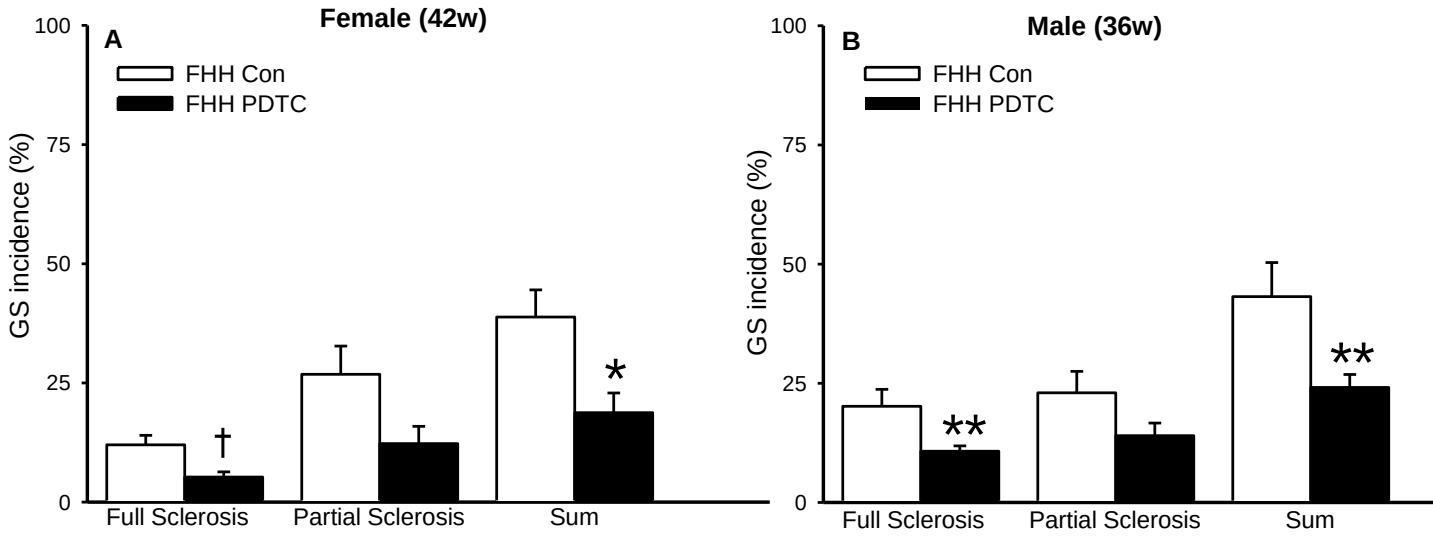

Female (42w)
A
FHH Con
FHH PDTC
GS incidence (%)
100
75
50
25
0
†
*
Full Sclerosis
Partial Sclerosis
Sum
Male (36w)
B
FHH Con
FHH PDTC
GS incidence (%)
100
75
50
25
0
**
**
Full Sclerosis
Partial Sclerosis
Sum

Figure 5

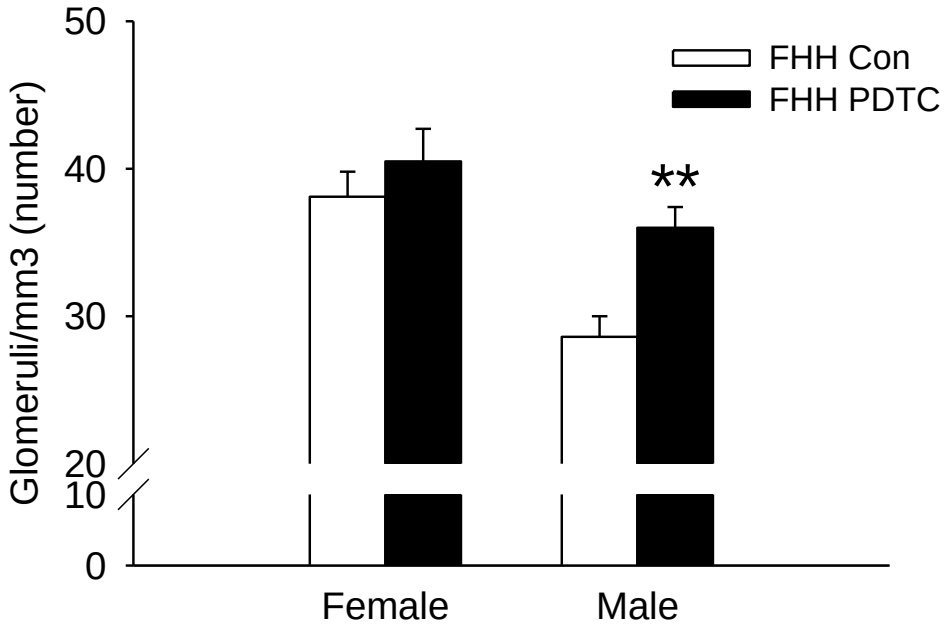

50
40
30
20
10
0
Glomeruli/mm3 (number)
FHH Con
FHH PDTC
**
Female
Male

Figure 6

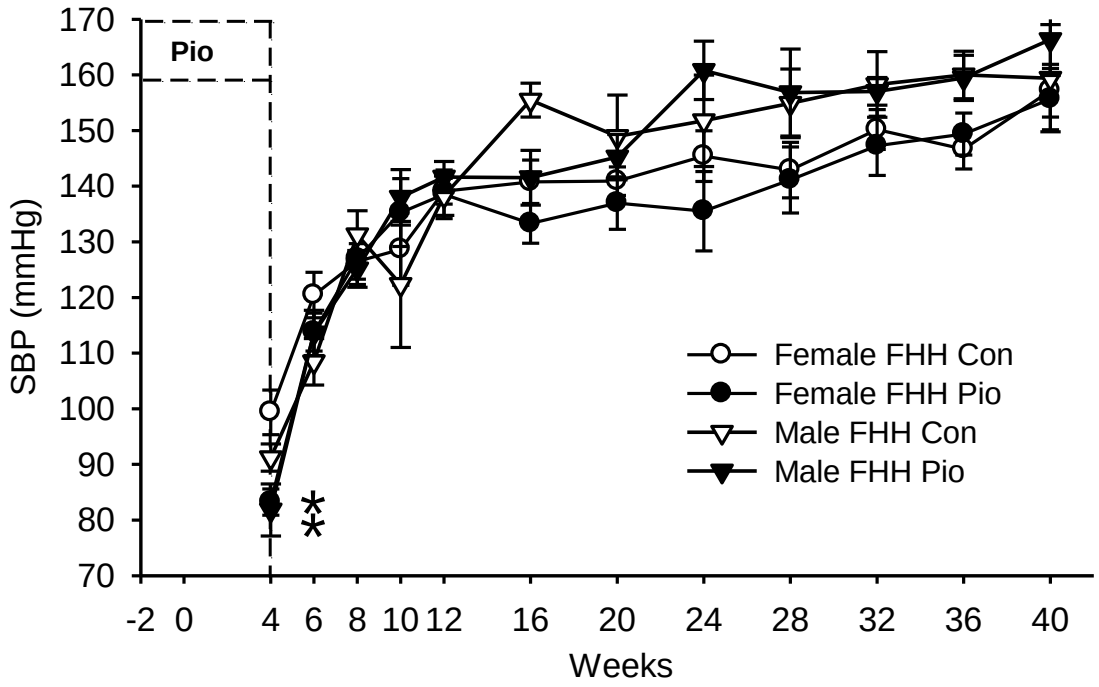

Pio
SBP (mmHg)
170
160
150
140
130
120
110
100
90
80
70
-2
0
4
6
8
10
12
16
20
24
28
32
36
40
Weeks
Female FHH Con
Female FHH Pio
Male FHH Con
Male FHH Pio
*
*

Figure 7

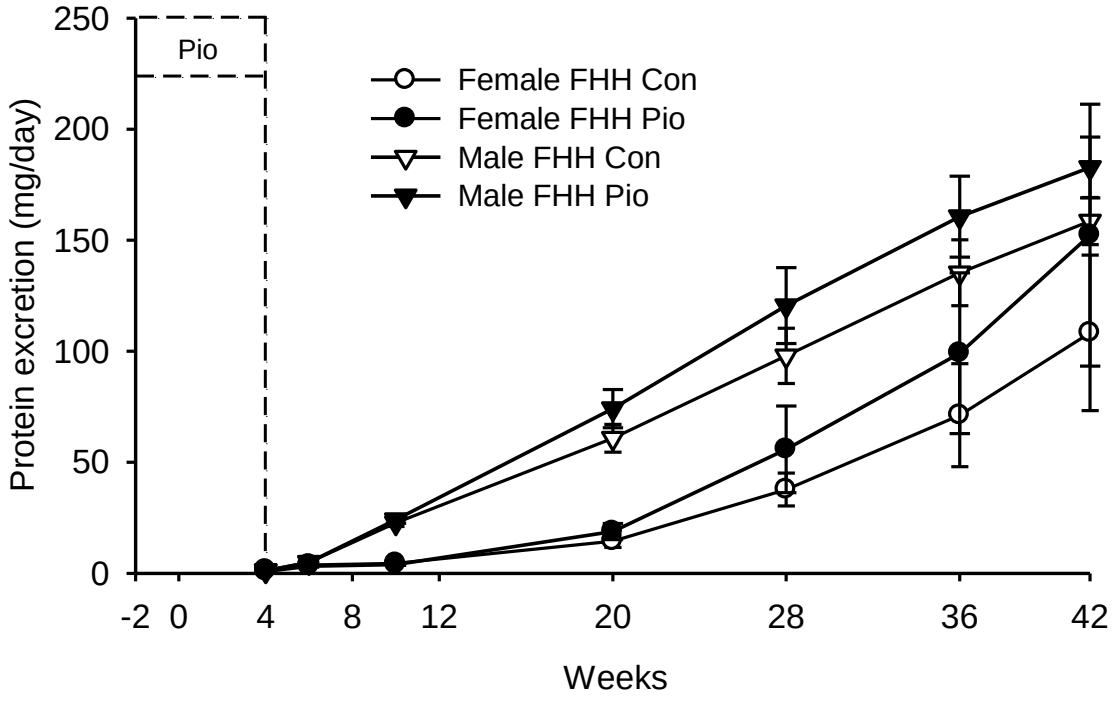
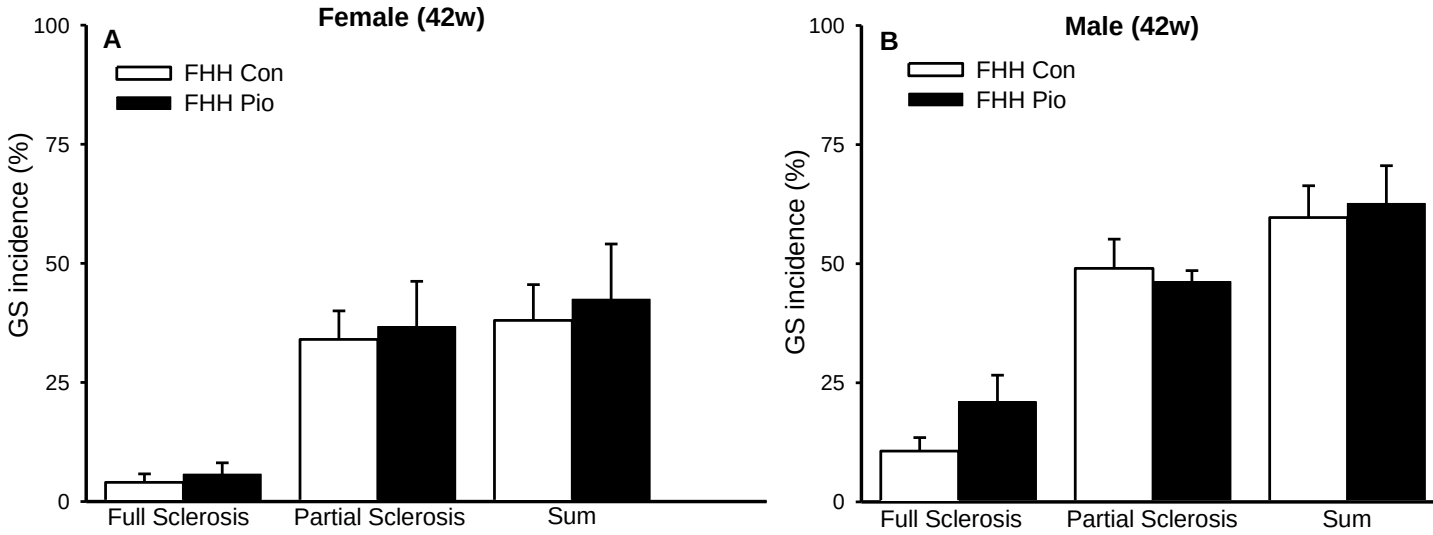

Pio
Female FHH Con
Female FHH Pio
Male FHH Con
Male FHH Pio
Protein excretion (mg/day)
250
200
150
100
50
0
-2
0
4
8
12
20
28
36
42
Weeks

Figure 8


Female (42w)
A
FHH Con
FHH Pio
GS incidence (%)
100
75
50
25
0
Full Sclerosis
Partial Sclerosis
Sum
Male (42w)
B
FHH Con
FHH Pio
GS incidence (%)
100
75
50
25
0
Full Sclerosis
Partial Sclerosis
Sum

Perinatal inhibition of NF-kappaB has long-term antihypertensive and renoprotective effects in Fawn-Hooded Hypertensive Rats

- Supplement -

Methodology Microarray

After the hybridization of the samples to Illumina BeadChips RatRef-12 the raw data set containing all probes of each BeadArray was received. Each sample consisted of approximately 1 million beads, interrogating about 23K unique probes encoding genes (n=22523), controls, housekeeping genes and negative controls (n=825). Each gene is probed with at least 30 beads. Before averaging the intensities of beads per probe, the outliers were removed, using the same approach to removing outliers as in BeadStudio. The removal of outliers was based on median ± 3x median absolute deviation (MAD) and all beads with intensities outside the area of median ± 3xMAD were removed.Outliers were frequent for each probe. In our hands close to 90% of the probes had outliers. Note that the outliers in "empty" beads, the negative controls (NC), were not removed, as they were considered to be background noise of the beads themselves. After removal of outliers (average 3 beads) the number of beads per probe averaged 39 beads.

After the test, the intensities of beads per probeare averaged for further data processing. Before the normalization procedure, the significance of a call from a gene was detected by applying a detection score that is dependent on the distribution of the intensities and the average intensity of the gene and NC (regardless of the number of NC). In this framework we performed the appropriate Student's t-testbetween the beads of a gene and the average intensity of all NC on the same array including testing (in)equality between two population variances in order to enhance reliability of the t-test. All probes with a significant call above the negative background were considered biologically active in the respective sample.

The software (T4Illumina) was written to process the raw data by averaging the intensities and determining the call of a gene, including determining the (un)equality of variance and many more. This software is available at request.

The averaged intensities, after removal of outliers, of the probes from all BeadArrays are Log_2-transformed and quantile normalized. Next the arrays were grouped accordingly and the average intensity per group was calculated. Finally the significance of the differences in intensities between the groups was calculated using Cyber t-test. Cyber-T is a statistics program designed specifically for microarray data (http://cybert.ics.uci.edu/). The

normalization, averaging, and statistics procedures can all be performed by the software *FlexArray* (http://gqinnovationcenter.com/services/bioinformatics/flexarray/index.aspx?l=e).

Supplementary Figure Legends

Supplementary Figure 1. Body weight in female (circles) and male (triangles) FHH Con (white) and FHH PDTC (black). Mean ± SEM, two-way ANOVA repeated measures, SNK post-hoc test. * $p<0.05$ vs. FHH con

Supplementary Figure 2. Glomerular density in female (panel A) and male (panel B) FHH Con (white bars) and FHH PDTC (black bars).Mean ± SEM, t-test. ** $p<0.01$ vs. FHH Con

Supplementary Figure 3. Body weight in female (circles) and male (triangles) FHH Con (white) and FHH Pio (black). Mean ± SEM, two-way ANOVA repeated measures, SNK post-hoc test.* $p<0.05$ vs. FHH con

Supplementary Figure 4. Systolic blood pressure in female (circles) and male (triangles) FHH Con (white)and FHH Pio (black). Mean ± SEM, two-way ANOVA repeated measures, SNK post-hoc test. * $p<0.05$ vs. FHH control

Supplementary Figure 5. Urinary protein excretion in female (circles) and male (triangles) FHH Con (white) and FHH Pio (black). Mean ± SEM, two-way ANOVA repeated measures, SNK post-hoc test.

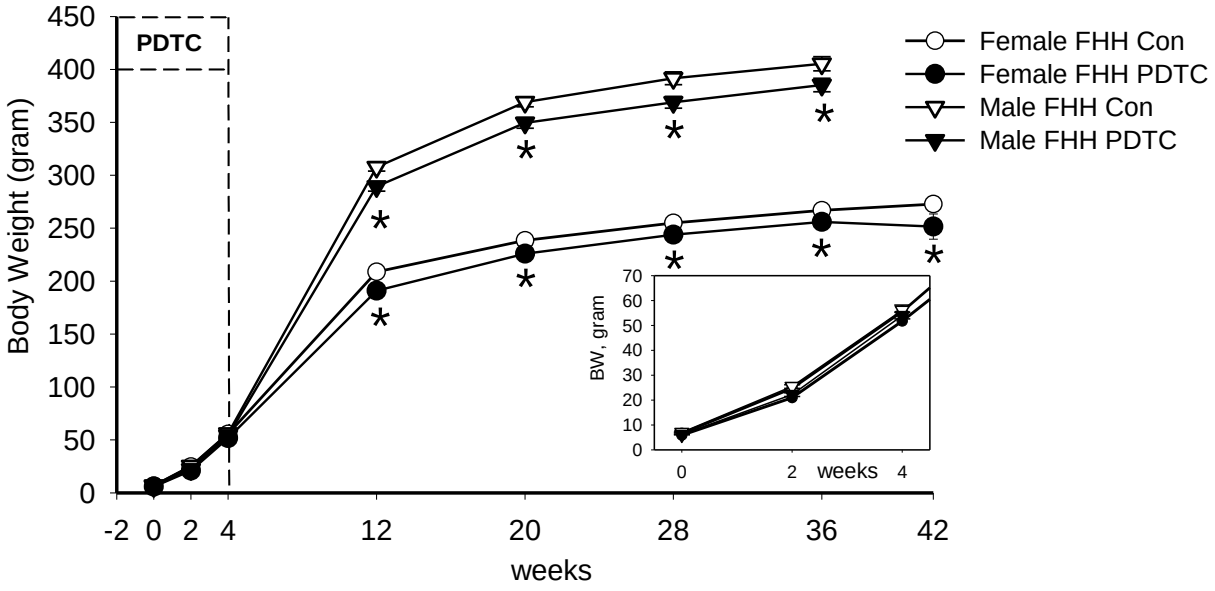
Supplementary Figure 6. Full, partial and sum glomerular sclerosis (GS) incidences in female (panel A) and male (panel B) FHH. Con (white bars) and FHH Pio (black bars).Mean ± SEM, t-test.

Supplementary Figure 7. Glomerular density in 10w (panel A) and 42w (panel B) female and male FHH Con (white bars) and FHH Pio (black bars).Mean ± SEM, t-test.

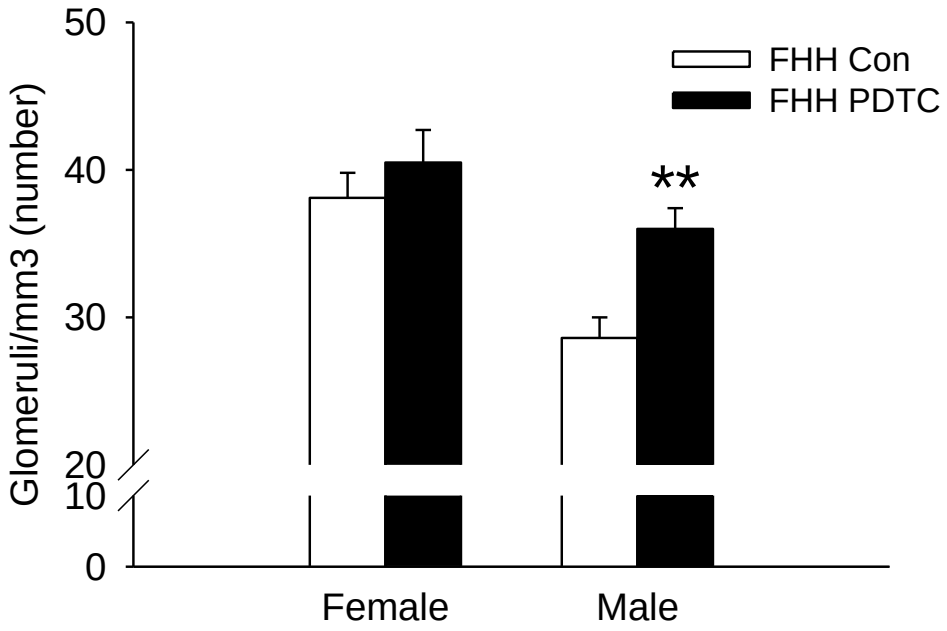
Supplementary Table 1. Differential expression (P<0.05vs. FHH Con set at 100%) at 2 days in the microarray.

		FHH PDTC versus FHH Con - 2 days old -	
Definition	**Symbol**	**Females**	**Males**
Regulation of NF-kB			
NFKB inhibitor interacting Ras-like 1	Nkiras1	126	122
PPAR			
peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Ppargc1a	149	145
Kidney-Specific			
nidogen 1	Nid1	151	168
notch 1	Notch1	134	132
nidogen 2	Nid2	129	138
nephrosis 1, congenital, Finnish type (nephrin)	Nphs1	119	121
protein phosphatase 4, regulatory subunit 1	Ppp4r1	124	118
Insulin-like Growth Factors			
pregnancy-associated plasma protein A	Pappa	159	137
pleiomorphic adenoma gene 1	Plag1	137	129
insulin-like growth factor binding protein 3	Igfbp3	125	129
insulin-like growth factor 2	Igf2	120	118
Angiogenesis			
SPARC related modular calcium binding 2	Smoc2	127	118
neuropilin 1	Nrp1; VEGF-165	125	127
vascular endothelial growth factor C	Vegfc	123	127
NO-related (cGMP, Adenylate cyclase, …)			
endothelin receptor type B	Ednrb	202	181
RAS p21 protein activator (GTPase activating protein) 1	Rasa1	156	151
protein kinase, cGMP-dependent, type 1	Prkg1	134	135
Cd47 molecule	Cd47	133	122
adenylate cyclase activating polypeptide 1	Adcyap1	130	122
A kinase (PRKA) anchor protein 13	Akap13	120	121
phosphodiesterase 9A	Pde9a	85	81
Structure-Related			
integrin, alpha 1	Itga1	211	173
palladin	Palld	206	160
collagen, type I, alpha 2	Col1a2	157	145
fibrillin 2	Fbn2	155	147
tensin 1	Tns1	155	149
transgelin	Tagln	155	125
protocadherin 17	Pcdh17	150	149
lumican	Lum	148	128
adducin 3 (gamma)	Add3	148	137
ARP2 actin-related protein 2 homolog (yeast)	Actr2	147	129
calcium/calmodulin-dependent protein kinase II gamma	Camk2g	146	133
collagen, type VIII, alpha 1	Col8a1	140	130
matrix metallopeptidase 14 (membrane-inserted)	Mmp14	138	140
collagen, type XIV, alpha 1	Col14a1	131	122
Rho-associated coiled-coil containing protein kinase 2	Rock2	129	128
collagen, type V, alpha 2	Col5a2	129	128
ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Atp2a2	127	121
collagen, type V, alpha 1	Col5a1	125	129
dynamin binding protein	Dnmbp	124	125
amine oxidase, copper containing 3 (vascular adhesion protein 1)	Aoc3	124	120
NCK interacting protein with SH3 domain	Nckipsd	122	120
ATPase, Ca++ transporting, plasma membrane 1	Atp2b1	121	120
core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1	C1galt1	121	123
collagen, type IV, alpha 3 (Goodpasture antigen) binding protein	Col4a3bp	121	120
fibulin 2	Fbln2	121	123
laminin, beta 1	Lamb1	117	126
tropomyosin 3, gamma	Tpm3	116	120
prominin 1	Prom1	116	122
collagen triple helix repeat containing 1	Cthrc1	115	124
calmodulin regulated spectrin-associated protein 1	Camsap1	113	118
deoxyribonuclease I	Dnase1	58	51

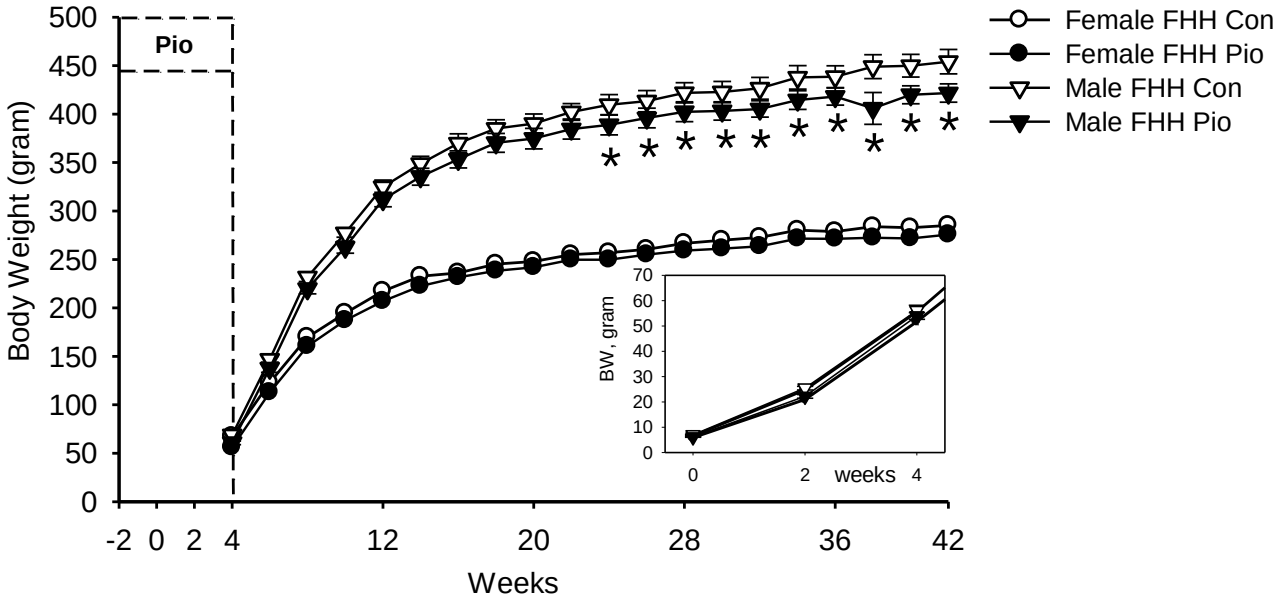
Supplementary Figure 1.



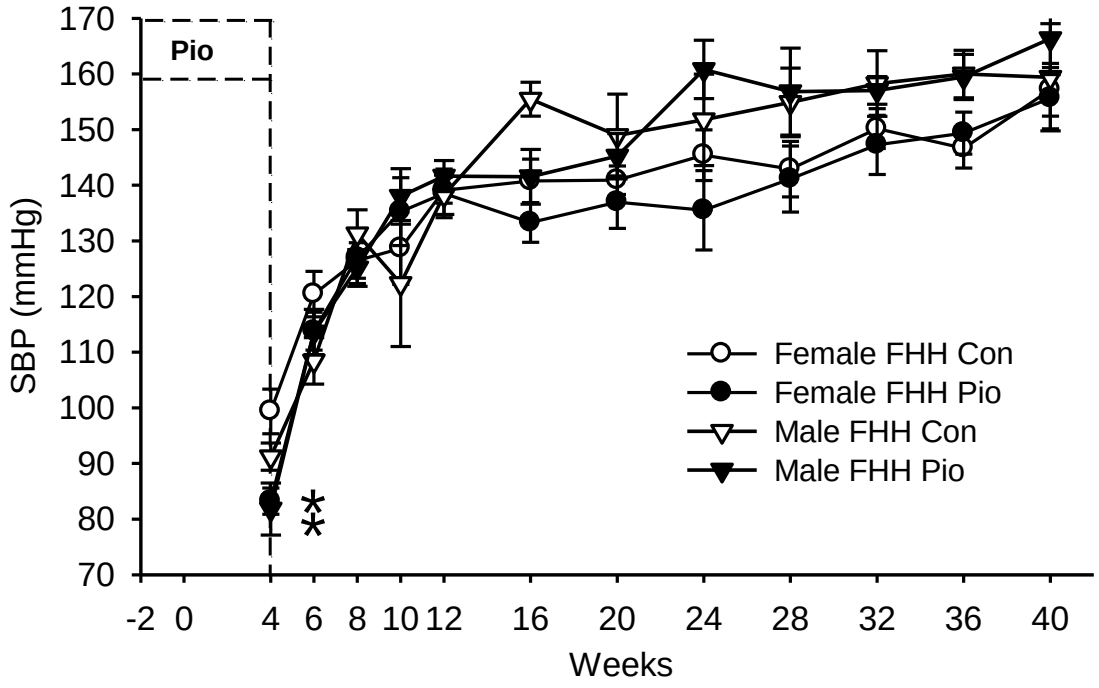
Supplementary Figure 2



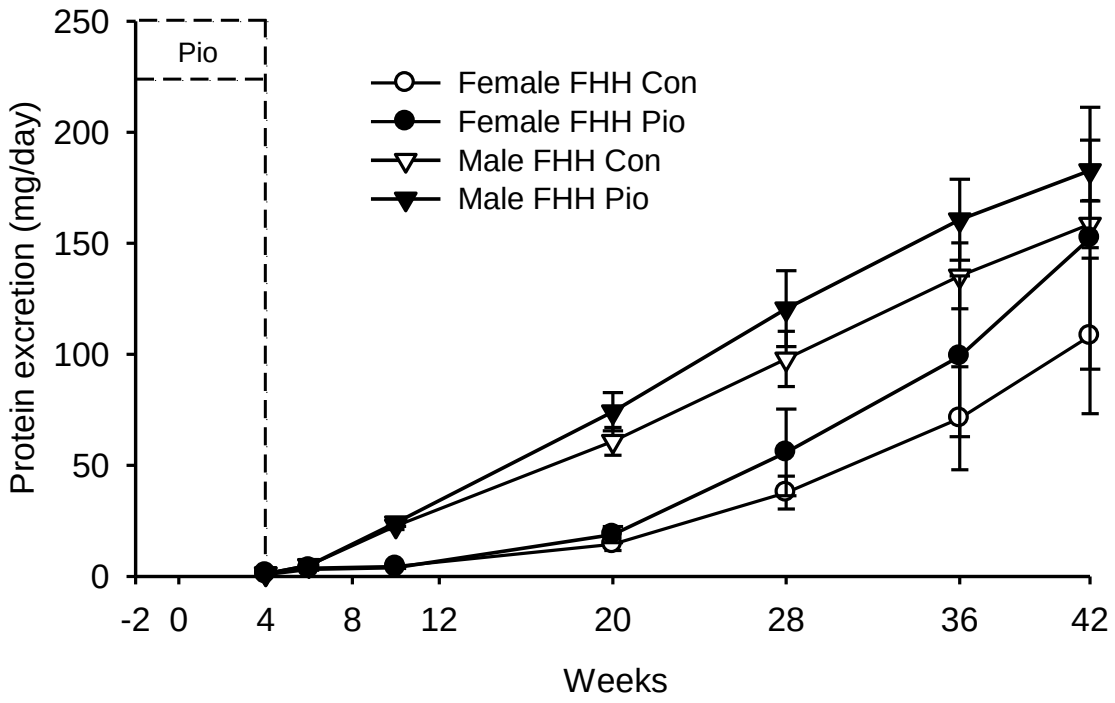
Supplementary Figure 3



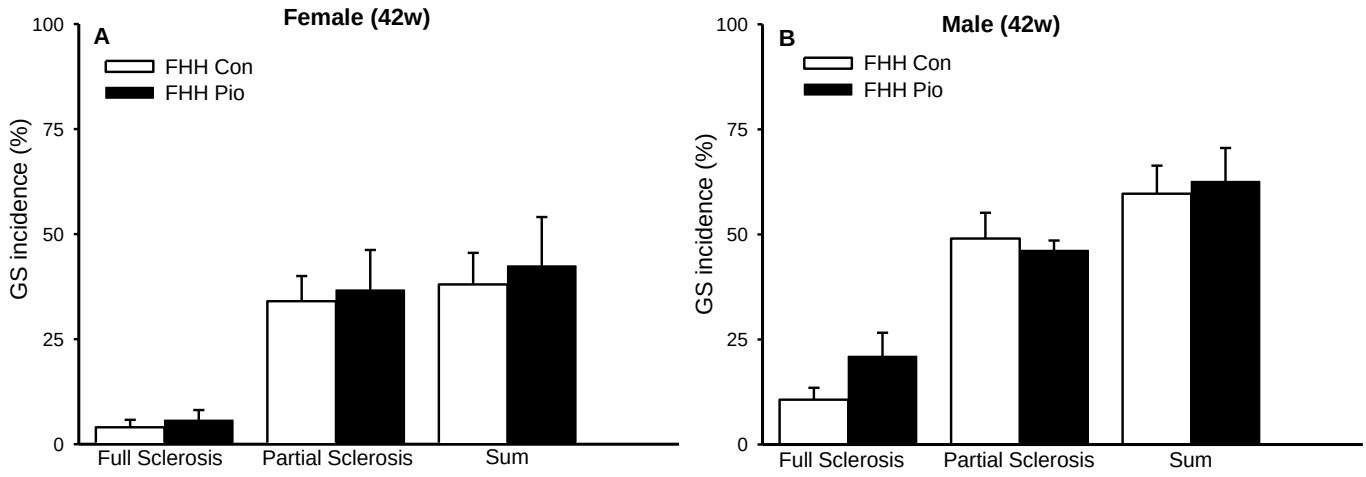
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7